Treatment of serious infections and septic shock

The invention relates to the use of rhesus theta-defensin 1 (RTD-1) for producing a medicament for the treatment and/or prophylaxis of patients with serious infections (bacteremias) including septic shock.

Sepsis is a severe, life-threatening syndrome resulting from an infection with bacteria at the systemic level (bacteremia) and further clinical findings according to the internationally valid definition (Madot, I. and Sprung, C.L. (2001), Int. Care Med. 27, S.3-S.9) which includes inter alia also the systemic inflammatory reaction of the body with subsequent organ failure. If a local infection by the pathogens breaks through the endogenous barriers (e.g. epithelial, endothelial, blood-brain barrier), a sepsis may develop from the bacteremia resulting therefrom. The microbes continuously enter the blood stream, and thus the whole body, from the septic focus (e.g. abscess, lung, gastrointestinal tract), which may also remain unidentified. Although the defense functions of the immune system impede reproduction of the microbes which have entered the blood and other immunological organs, they are usually not completely destroyed. In addition, the attack on the microbes by the immune system, and also therapies with certain antibiotics, result in the release of bacterial products such as lipopolysaccharide (LPS), lipoteichoic acid (LTA) and the like (Nau, R. and Eiffert, H. (2002), Clin. Microbiol. Rev. 15, 95-110).

Sepsis is characterized by fever, hypotension and so-called shock symptoms (e.g. shock lung, renal failure, gastrointestinal bleeding; generally referred to as multiorgan failure). These different symptoms are the clinical signs of pathophysiological processes brought about by the microbes themselves or their products, e.g. endotoxins, hemolysins or pyrogens. Further causes which may also occur are pathological states such as severe burns, trauma or acute pulmonary changes with subsequent or simultaneous colonization by bacteria, fungi or viruses.

Shock symptoms are also found in these cases, but only in some cases is direct diagnosis of the bacteria or other pathogens possible.

The release of bacterial products or the bacteria themselves lead to a response by the body through the immune system. Immune system factors intrinsic to the patient are in these cases both protective and harmful, depending on the concentration, site of action and the like. Endogenous factors released as a response of the body to external stimuli interact in a complex fashion in the tissues and thus lead in some circumstances to the pathological state of sepsis. These events are initiated by definition through the presence of bacteria and/or the presence of bacterial products such as LPS/LTA and others. This leads to the release of, for example, tumor necrosis factor α, interleukin-1 (IL-1), interleukin-6 (IL-6) and interleukin-8 (IL-8), and factors which influence coagulation (e.g. platelet activating factor, PAF) and factors which intervene to regulate the resulting inflammatory event (such as, for example, prostaglandins, leukotrienes, interleukin-10). An overreation of the body, and especially of the immune system, results in the development far from the orginally protective and infection-repelling response, of the clinical feature of bacteremia and subsequently of sepsis (Cohen, J. (2002), Nature 420, 885-891). Cytokine analyses in septic patients show a significant correlation with elevation of, in particular, IL-6, IL-8 and TNF-α and an increased mortality (Rodriguez-Gaspar M. et al. (2001), Cytokine 15, 232-236).

Although in principle virtually all microorganisms may induce bacteremia and thus subsequently sepsis, the percentage distribution of the microbes depends on the age of the patient, the basic disorder, the primary infectious focus and the like (Knaus, W.A. et al. (1985), Crit. Care. Med. 13, 818-829). Leading microbes are both Gram-positive and Gram-negative bacteria, and fungi and yeasts (Llewelyn, M and Cohen, J. (2001), Int. Care Med. 27, S.10-S.32).

Therapeutic methods and active ingredients which prevent the release of such microbial products or bind and neutralize such products or influence endogenous functions and factors or lead to death of the microbes are suitable for the treatment of symptoms of the abovementioned diseases.

Substances and methods leading to a reduction in the number of microbes (e.g. antibiotics), as well as substances having a circulation-influencing effect, can be utilized therapeutically (Forth, Henschler, Rummel; Allgemeine und spezielle Pharmakologie und Toxikologie; Urban & Fischer Verlag (2001), Munich). The standard therapy for the disease described above are antibiotics together with substances or solutions which stabilize the circulation and influence coagulation.

A further approach is to influence the pathological state by immunomodulatory treatments in order to prevent or at least ameliorate an excessive response of the body to bacteria or bacterial products and thus avert organ failure (Zanotti, S. et al. (2002), Expert Opin. Investig. Drugs 11, 1061-1075).

In this connection, soluble mediators of the immune system which are supplied as therapy are also of particular interest. These mediators include inter alia the so-called defensins, molecules having antibacterial, antifungal or else antiviral properties (Kagan, B.L. et al. (1994), Toxicology 87, 131-149; Hancock R.E.W. and Scott M.G. (2000), PNAS 16, 8856-8861). In addition, these molecules, which are small peptides, have immune system-modulatory properties (Hancock R.E.W. and Scott M.G. (2000), PNAS 16, 8856-8861). They bring about or prevent for example the release of further mediators.

Even with optimal treatment according to the current therapeutic standard, the mortality in cases of sepsis and septic shock is up to 50% of the patients (Cohen, J. (2002), Nature 420, 885-891). Additional therapeutic treatments are therefore urgently necessary. However, in various approaches followed for the treatment of patients with severe bacteremias, sepsis and/or septic shock it has emerged, even

with newer therapies, that intervention in a disease process at one point is unsuccessful or only partly successful. A combination of various therapeutic approaches proves to be the most promising in the treatment of diseased patients (Anel, R.L. and Kumar, A. (2001) Expert. Opin. Investig. Drugs 10, 1471-1485).

A defensin from immune cells of the rhesus monkey (rhesus theta-defensin 1; RTD-1) has been isolated and its properties, its broad antibacterial effect, even on non-growing bacteria, and its antifungal effect has been described in detail (Tang et al. (1999), Science 286, 498-502; Tran et al. (2002), J. Biol. Chem. 277, 3079-3084) and a patent application has been made therefor (WO 00/68265). RTD-1 is distinguished from other defensins or cationic peptides by some characteristic and defining properties. Firstly, RTD-1 is a small circular peptide and thus, in contrast to other defensins, stable to breakdown and degradation. RTD-1 shows no dependence of the effect on salts in the medium, and thus no loss of action in the presence of physiological concentrations of various salts such as, for example, NaCl, KCl etc. (Muhle et al. (2001), Biochemistry 40, 5777-5785; Tang et al. (1999), Science 286, 498-502), nor any loss of action in the presence of human serum (WO 00/68265). In addition, no hemolytic effect has been found.

In the invention described herein, surprisingly, a further effect of RTD-1 is found in states of bacteremia with subsequent sepsis and septic shock, and such pathological states after exposure to bacterial products such as, for example, LPS. In this connection, an effect has been found on 4 different pathological states which are relevant for the syndrome of severe bacteremias, sepsis and septic shock. RTD-1 intervenes in the pathological process by 1. showing an antimicrobial effect on various pathogens, 2. showing a neutralizing effect on bacterial products such as LPS or LTA (effect on products of Gram-positive as well as Gram-negative bacteria), which also permits prophylactic therapy, 3. having an immunomodulatory effect in the sense of mediator modulation, and 4. having a regulated anticoagulant effect. The combined effect on various parameters relevant to the disease results in an unambiguously improved success of therapy together with simplified therapy. In

addition, therapy with RTD-1 does not rule out current standard therapies (e.g. antibiotics, circulation-stabilizing substances) and allows combination therapy.

Therapy with RTD-1 leads to increased survival of mice with severe bacteremia after administration of living bacteria, in particular both after infection with Gram-positive and Gram-negative bacteria. Moreover, surprisingly, no dependence is to be found with the minimum inhibitory concentration of RTD-1 on the bacterium used.

Therapy of mice with symptoms of septic shock after administration of LPS or SEB also shows a distinctly increased survival with RTD-1 therapy. Cytokine analyses in the serum or plasma of the animals show a regulation of the release of soluble mediators. It is moreover possible to demonstrate a reduction of pro-inflammatory cytokines (such as, for example, TNF-α, IL-6, MIF) and an increase in regulatory factors (such as, for example, IFN-γ, IL-10). Comparable results are found in human whole blood, there being a reduction in the levels of pro-inflammatory cytokines and chemokines.

Under the influence of RTD-1 there is a dose-dependent increase in the clotting time of human plasma and human whole blood. RTD-1 thus shows an influence on the coagulation parameters in human blood without an additional influence on coagulation due to bacterial products such as, for example, LPS or LTA.

The present invention therefore relates to the use of theta-defensin from the rhesus monkey for producing a medicament for the treatment and/or prophylaxis of bacteremias and/or sepsis.

In this connection, the disease-causing agents may be Gram-positive bacteria, Gram-negative bacteria, bacterial products, viruses or yeast fungi.

The invention further relates to the use of RTD-1 for producing a medicament for binding bacterial products such as LPS and/or LTA.

The invention further relates to the use of RTD-1 for producing a medicament for the treatment of pathological states characterized by changes in blood clotting.

The applications mentioned can moreover be combined with standard antibiotics or standard antimycotics.

Examples

Example 1

Test for antibacterial effect in vitro

To determine the minimum inhibitory concentration, bacteria of various strains are exposed to graded concentrations of RTD-1, using a 1:2 dilution series. Determination takes place according to the principles of the NCCLS (see documentation: Methods for dilution antimicrobial susceptibility tests for bacteria, NCCLS document M7-A5, Vol. 20 No. 2).

Table 1: Minimum inhibitory concentrations of RTD-1, vancomycin and ampicillin for various bacterial species determined by the NCCLS method. The table indicates the concentration of the respective compounds which showed unambiguous inhibition of the growth of the bacteria.

Bacterium	MIC [mg/L]		
	RTD-1	Vancomycin	Ampicillin
S. aureus MSSA	1	<0.125	0.5
S. aureus MRSA	8	1	>64
E. faecalis	2	2	2
E. faecium	1	0.5	4
E. faecium VRE	0.5	>64	64
S. pneumoniae	64	0.25	0.125
E. coli	16	>64	8
P. aeruginosa	32	>64	>64
K. pneumoniae	8	>64	2
S. typhimurium	8	>64	4

Table 1: Minimum inhibitory concentration of RTD-1, vancomycin and ampicillin for various bacterial species

Example 2

***In vivo* investigations in disease-relevant animal models**

Bacterial suspensions are administered intraperitoneally (i.p.) to CFW-1 mice. The mice are purchased from Harlan. After 30 min, the animals are then treated intravenously (i.v.) with RTD-1 in various doses. The survival of the animals with and without therapy reveals the success of therapy.

Table 2: Survival of mice after i.p. infection with *S. aureus* in the bacterema model and therapy with 0.1, 1 and 10 mg/kg RTD-1 i.v.. The mice are infected i.p. with 1.68×10^7 colonies of *S. aureus* ATCC Smith and, after 30 min, are treated with the stated dose i.v.. The table indicates the % of survivors from n= 6 mice on day 5 after infection.

Dose	% Survival
No RTD-1 (control)	17
0.1 mg/kg	67
1 mg/kg	83
10 mg/kg	83

Table 2: Survival of *S. aureus*-infected mice with and without RTD-1 therapy

Table 3: Survival of mice after i.p. infection with *S. pneumoniae* in the bacterema model and therapy with 0.1, 1 and 10 mg/kg RTD-1 i.v.. The mice are infected i.p. with 3×10^3 colonies of *S. pneumoniae* L3TV and, after 30 min, are treated with the stated dose i.v.. The table indicates the % of survivors from n= 6 mice on day 5 after infection.

Dose	% Survival
No RTD-1 (control)	17
0.1 mg/kg	50
1 mg/kg	67
10 mg/kg	33

Table 3: Survival of *S. pneumoniae*-infected mice with and without RTD-1 therapy

Table 4: Survival of mice after i.p. infection with *E. coli* in the bacterema model and therapy with 0.1, 1 and 10 mg/kg RTD-1 i.v.. The mice are infected i.p. with 1.68×10^7 colonies of *E. coli* Neumann and, after 30 min, are treated with the stated dose i.v.. The table indicates the % of survivors from n= 6 mice on day 5 after infection.

Dose	% Survival
No RTD-1 (control)	33
0.1 mg/kg	50
1 mg/kg	83
10 mg/kg	83

Table 4: Survival of *E. coli*-infected mice with and without RTD-1 therapy

Example 3

In addition, LPS is injected i.p. into mice and, at various times before and after the LPS administration, RTD-1 is administered i.v. in various dosage in order to simulate a septic shock. The survival of the animals is the measure of the success of therapy in the model of septic shock.

Table 5: Survival of mice after i.p. administration of 20 mg/kg LPS from *S. typhimurium* (Sigma) and therapy with 0.1, 1 and 10 mg/kg RTD-1 i.v. at various times before and after LPS administration. The table indicates the % of survivors from n= 5 mice on day 5 after LPS administration.

Dose	% Survival				
	-2h	-1h	+0.1h	+1h	+2h
No RTD-1 (control)			0		
0.1 mg/kg	100	100	100	100	80
1 mg/kg	100	100	100	100	100
10 mg/kg	80	80	100	80	80

Table 5: Survival of LPS-treated mice with and without RTD-1 therapy

Example 4

Investigations on the binding of bacterial products

The binding of RTD-1 to lipopolysaccharide (LPS) and lipoteichoic acid (LTA) is investigated in a binding mixture in vitro. This entails use of dansyl-polymyxin B (Moore et al. (1986), Antimicrob. Agents Chemotherap. 29, 496-500) which fluoresces after binding to LPS or LTA. Competition with RTD-1 results in a decrease in the fluorescence and, resulting therefrom, a determination of the relative inhibition of the binding of dansyl-polymyxin B.

From the inhibition it is possible in turn to calculate a relevant affinity of RTD-1 for binding of bacterial products.

Table 6: LPS and LTA binding by RTD-1 and polymyxin B after incubation for 4 hours. The concentration at which the fluorescence of dansyl-polymyxin B is reduced by 50% is indicated.

Test substance	50% LPS binding at µM	50% LTA binding at µM
RTD1	0.001	0.1
Polymyxin B	4.9	0.01

Table 6: LPS and LTA binding by RTD-1 and polymyxin B.

Example 5

The membrane permeability of bacteria under influence of RTD-1 is investigated using a fluorescence method (Silvestro et al. (2000), Antimicrob. Agents Chemotherap. 44, 602-607). This permits assessment of the potential of RTD-1 in relation to damage to the cell membrane of bacteria and thus on the release of bacterial products.

Table 7: Membrane potential change under the influence of RTD-1 and polymyxin B after exposure of *S. aureus* bacteria for 10 minutes. The concentration leading to a 50% change in the fluorescence signal is shown.

Test substance	50% change in fluorescence at µM
RTD1	3.9
Polymyxin B	>75

Table 7: Membrane potential change under the influence of RTD-1 and polymyxin B.

Example 6

In addition, the influence of RTD-1 on bacterial cell wall synthesis is investigated. This entails RTD-1 being added to a membrane fraction from *E. coli* (as example of Gram-negative bacteria) or from *Bacillus megaterium* (as example of Gram-positive bacteria) and the necessary substrates, and the inhibitory activity being determined (Chandrakala, B. *et al.* (2001) Antimicrob. Agents Chemother. 45, 768-775). This approach determines the incorporation of radioactive precursors in high molecular weight peptidoglycan via binding to wheat germ agglutinin. An influence of RTD-1 on bacterial cell wall synthesis provides substantial information about an effect on the bacterium (e.g. lysis) and especially in connection with the release of bacterial products, which is a substantial precondition for assessing the therapeutic potential of RTD-1 in serious infections including septic shock.

Table 8: Inhibition of cell wall synthesis by RTD-1, ampicillin (Sigma), a lactam antibiotic, chloramphenicol (Sigma), a protein biosynthesis inhibitor and vancomycin (Sigma), a glycopeptide antibiotic. The inhibition of the binding to wheat germ agglutinin of radioactive precursors in high molecular weight peptide or glycan is shown. Only substances influencing cell wall biosynthesis but not inhibiting protein biosynthesis show inhibition in this approach.

Test substance	Gram-negative 50% inhibition at µM	Gram-positive 50% inhibition at µM
Ampicillin	8.8	5.0
Chloramphenicol	>100	>100
Vancomycin	4.4	0.85
RTD1	7.2	5.7

Table 8: Inhibition of cell wall synthesis in Gram-negative and Gram-positive bacteria and bacterial lysates by RTD-1, ampicillin, vancomycin and chloramphenicol.

Example 7

Investigation of the influence on the prothrombin time (PT)

This parameter is employed to determine disturbances in the extrinsic system of blood clotting. The prothrombin time is determined on citrated plasma after addition of calcium and tissue factor. For this purpose, blood is taken from healthy people of both genders in collecting tubes with citrate (Monovetten, Sarstedt, Nürnbrecht, Germany) and the plasma is obtained after centrifugation. Samples of this plasma are incubated with various concentrations of the test compounds at 37°C for 10 minutes. Then thromplastin (Recombiplastin, OrthoDiagnostic Systems, Neckargemünd, Germany) is added in order to initiate the extrinsic pathway of blood clotting. This mixture is mixed and the clotting time is determined in an apparatus for determining coagulation (Coagulometer, Amelung, KC 4A micro).

Investigations of the influence on the partial thromboplastin time (aPTT)

This parameter is employed to determine disturbances in the intrinsic system of blood clotting. The prothrombin time is determined on citrated plasma after addition of an activator and phospholipid. For this purpose, blood is taken from healthy people of both genders in collecting tubes with citrate (Monovetten, Sarstedt, Nürnbrecht, Germany) and the plasma is obtained after centrifugation. Samples of this plasma are incubated with various concentrations of the test compounds at 37°C for 10 minutes. The activator kaolin and phospholipid (aPTT Reagenz, Diagnostica Stago, Asnieres, France) is then added. Coagulation is started by adding 0.025 M calcium chloride to this mixture. This mixture is mixed and the clotting time is determined in an apparatus for determining coagulation (Coagulometer, Amelung, KC 4A micro).

Table 10: Influence on the coagulation parameters aPTT, PT, and the clotting time of human whole blood under the influence of RTD-1. The clotting time in seconds after addition of RTD-1 is shown.

RTD-1 concentration in µg/ml	Clotting time [sec] aPTT	PT	Whole blood
0 (control)	33.9	14.55	351.94
5	35.4	15.7	368.2
10	36.6	21.2	375.2
30	43.4	51.3	475.4
50	112.7	67.0	598.3
100	297 (stopped)	102.8	1000 (stopped)

Table 10: Influence on the coagulation parameters aPTT, PT, and the clotting time of human whole blood under the influence of RTD-1.

Example 8

***In vivo* cytokine investigations in disease-relevant animal models**

At various times after LPS or SEB administration to mice, blood samples are obtained by exsanguination of the animals. Plasma samples are drawn from these blood samples and subjected to a cytokine analysis. The amount of cytokines in the plasma is determined quantitatively using the CBA methods (CBA system, BectonDickinson, Heidelberg, Germany).

Table 11: Cytokine modulation after therapy with RTD-1 in the mouse model of septic shock after administration of LPS. The maximum percentage change compared

with the untreated LPS control is indicated. Negative values indicate a reduction, and positive values an increase, in the amount of cytokines in the plasma.

Cytokine	Change as % of the untreated LPS control on intravenous therapy with		
	RTD-1 0.1 mg/kg	RTD-1 1 mg/kg	RTD-1 10 mg/kg
TNF-α	-47.5	-41.1	-12.4
IL-6	-41.6	-5.8	
IL-10	+157	+159	+241
IFN-γ	+53	+138	+69

Table 11: Cytokine modulation after therapy with RTD-1 in the mouse model of septic shock after administration of LPS.

Table 12: Cytokine modulation after therapy with RTD-1 in the mouse model of septic shock after administration of SEB. The maximum percentage change compared with the untreated SEB control is indicated. Negative values indicate a reduction, and positive values an increase, in the amount of cytokines in the plasma.

Cytokine	Change as % of the untreated SEB control on intravenous therapy with		
	RTD-1 0.1 mg/kg	RTD-1 1 mg/kg	RTD-1 10 mg/kg
TNF-α	-32.3	-29.7	-39.3
IFN-γ	+8.2	+4.6	+28.6

Table 12: Cytokine modulation after therapy with RTD-1 in the mouse model of septic shock after administration of SEB.

Example 9

***Ex vivo* cytokine investigations in infected human blood**

Blood is taken from healthy donors and infected in vitro with bacteria. Both Gram-positive pathogens (*S. aureus*) and Gram-negative pathogens (*E. coli*) are used in this case. After addition of the pathogens, samples of the infected and of the infected and treated blood are taken at defined times, and the plasma is obtained by centrifugation. The amount of cytokines in the plasma is determined quantitatively by the CBA methods (CBA system, BectonDickinson, Heidelberg, Germany). In addition, MIF is analyzed by the ELISA technique (human MIF ELISA System, R&D Systems Inc., Minneapolis, USA).

Table 13: Cytokine modulation after therapy with RTD-1 in the infection model with human whole blood after administration of *S. aureus*. The maximum percentage change in pro-inflammatory mediators compared with the untreated infection control is indicated. Negative values indicate a reduction, and positive values an increase, in the amount of cytokines in the blood culture.

Cytokine	Change as % of the untreated infection control on therapy with		
	RTD-1 0.1 µg/ml	RTD-1 1 µg/ml	RTD-1 10 µg/ml
TNF-α	-79	-85	-43
IL-1ß	-71	-73	-10
IL-6	-58	-73	-3
IL-8	-17	-25	-3
MIF	-48	-44	-4

Table 13: Cytokine modulation after therapy with RTD-1 in the infection model with human whole blood after administration of *S. aureus*.

Table 13: Cytokine modulation after therapy with RTD-1 in the infection model with human whole blood after administration of *E. coli*. The maximum percentage change compared with the untreated infection control is indicated. Negative values indicate a reduction, and positive values and increase, in the amount of cytokines in the blood culture.

Cytokine	Change as % of the untreated infection control on therapy with	
	RTD-1 10 µg/ml	RTD-1 100 µg/ml
TNF-α	-8	-4
IL-6	-43	-20
MIF	-77	-41

Table 14: Cytokine modulation after therapy with RTD-1 in the infection model with human whole blood after administration of *E. coli*

Formulations

RTD-1 can be converted in a known manner into the usual formulations such as tablets, coated tablets, pills, granules, aerosols, syrups, emulsions, suspensions and solutions, using inert, nontoxic, pharmaceutically suitable carriers or solvents. In these cases, the therapeutically effective compound is to be present in each case in a concentration of from 0.5 to 90% by weight of the complete mixture, i.e. in amounts which are sufficient to achieve the indicated dosage range.

The formulations are produced for example by extending the active ingredients with solvents and/or carriers, where appropriate using emulsifiers and/or dispersants, it being possible for example when water is used as diluent where appropriate to use organic solvents as auxiliary solvents.

Administration takes place in a conventional way, preferably intravenously, transdermally, orally or parenterally, in particular orally or intravenously. However, it can also take place by inhalation through the mouth or nose, for example with the aid of a spray, or topically through the skin.

It has generally proved to be advantageous to administer amounts of about 0.001 to 10 mg/kg, on oral administration preferably about 0.005 to 3 mg/kg, of bodyweight to achieve effective results.

It may be necessary where appropriate to deviate from the stated amounts, in particular as a function of the bodyweight or the nature of the administration route, the individual response to the medicament, the nature of its formulation and the time or interval over which administration takes place. Thus, it may be sufficient in some cases to make do with less than the aforementioned minimal amount, whereas in other cases the stated upper limit must be exceeded. If larger amounts are administered, it may be advisable to divide these into a plurality of single doses over the day.

Literature list:

Chandrakala, B. *et al.* (2001) Antimicrob. Agents Chemother. 45, 768-775

Forth, Henschel, Rummel (2001), Allgemeine und spezielle Pharmakologie und Toxikologie; Urban&Fischer Verlag, Munich

Moore et al. (1986), Antimicrob. Agents Chemotherap. 29, 496-500

Muhle et al. (2001), Biochemistry 40, 5777-5785

Silvestro et al. (2000), Antimicrob. Agents Chemotherap. 44, 602-607

Tang et al. (1999), Science 286, 498-502

Tran et al. (2002), J. Biol. Chem. 277, 3079-3084

List of abbreviations:

ATCC	American Type Culture Collection
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MIC	Minimum inhibitory concentration
NCCLS	National Committee for Clinical Laboratory Standards
RTD	Rhesus theta defensin